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Evolutionary Origin of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

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ABSTRACT Several lines of evidence indicate that the most primitive staphylococcal species, those of the *Staphylococcus sciuri* group, were involved in the first stages of evolution of the staphylococcal cassette chromosome *mec* (SCC*mec*), the genetic element carrying the β-lactam resistance gene *mecA*. However, many steps are still missing from this evolutionary history. In particular, it is not known how *mecA* was incorporated into the mobile element SCC prior to dissemination among *Staphylococcus aureus* and other pathogenic staphylococcal species. To gain insights into the possible contribution of several species of the *Staphylococcus sciuri* group to the assembly of SCC*mec*, we sequenced the genomes of 106 isolates, comprising *S. sciuri* ($n = 76$), *Staphylococcus vitulinus* ($n = 18$), and *Staphylococcus fleurettii* ($n = 12$) from animal and human sources, and characterized the native location of *mecA* and the SCC insertion site by using a variety of comparative genomic approaches. Moreover, we performed a single nucleotide polymorphism (SNP) analysis of the genomes in order to understand SCC*mec* evolution in relation to phylogeny. We found that each of three species of the *S. sciuri* group contributed to the evolution of SCC*mec*: *S. vitulinus* and *S. fleurettii* contributed to the assembly of the *mec* complex, and *S. sciuri* most likely provided the mobile element in which *mecA* was later incorporated. We hypothesize that an ancestral SCC*mec* III cassette (an element carried by one of the most epidemic methicillin-resistant *S. aureus* clones) originated in *S. sciuri* possibly by a recombination event in a human host or a human-created environment and later was transferred to *S. aureus*.

KEYWORDS SCC*mec*, beta-lactam resistance, evolution, *mecA*, staphylococci

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The most important mechanism of resistance to β-lactam antibiotics is associated with the *mecA* gene, which encodes an extra penicillin-binding protein, called PBP2a, that has low affinity to virtually all β-lactam antibiotics (1). The *mecA* gene is carried on a structurally complex mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) that can also transport determinants of resistance to other antimicrobials, virulence determinants, and other genes important for bacterial survival under stress conditions (2). Thus, in a single event of genetic acquisition, SCC*mec* can turn susceptible staphylococci into virulent multidrug-resistant pathogens, well adapted to thrive in an infection situation, particularly in the hospital environment.

The advantage for bacteria to carry this element is indicated by the wide distribution of SCC*mec* among both nosocomial and community staphylococcal populations (3). Dissemination of SCC*mec* among *Staphylococcus aureus* strains constitutes a real public health threat worldwide (4) due to the associated complications in treatment and disease outcome.

SCC*mec* has a modular structure: it is composed of two essential elements, the *mec* complex, consisting of *mecA* and its regulators (*mecR1* and *mecI*), and the *ccr* complex, containing cassette chromosome recombinase (*ccr*) genes that ensure the mobility of the cassette (2, 5). SCC*mec* may also carry insertion sequences (IS), transposons, and plasmids, as well as housekeeping genes inside the so-called joining regions (J1 to J3). SCC*mec* inserts at a specific site on the chromosome, downstream of *orfX* (2) (recently renamed *rlmH*), which encodes an rRNA methyltransferase (6). The *ccr* genes were previously shown to be serine recombinases able to carry out both excisive and integrative recombination of SCC elements. The integration of SCC*mec* occurs through *ccr*-mediated recombination between a sequence in the genome (designated *attB*) and a specific site in the circularized SCC*mec* element (designated *att*SCC) (2, 7). During SCC*mec* insertion, a new pair of sites, referred to as *attL* and *attR*, flanking the element, is generated. During SCC*mec* excision, the reverse occurs: *attR* and *attL* sites are recombined to regenerate the original sites, *attB* and *att*SCC (2, 8).

Several lines of evidence suggest that SCC*mec* evolution occurred in the most primitive group of *Staphylococcus* species, the *Staphylococcus sciuri* group. Previous studies showed that *mecA1*, a ubiquitous gene in *S. sciuri* with 80% nucleotide identity to *S. aureus mecA*, was the most probable evolutionary precursor of *mecA* (9, 10). Follow-up studies showed that *mecA1* encoded an *S. sciuri* penicillin-binding protein that participates in cell wall biosynthesis and that was able to express resistance in both *S. sciuri* and *S. aureus* (11, 12). Moreover, other homologues closer to *mecA* than *mecA1* along evolutionary lines were also identified in other species of the *S. sciuri* group, namely, *mecA2* (90% identity) in *S. vitulinus* and *mecA* (99% identity) in *S. fleurettii*, suggesting a vertical evolution of the gene along phylogeny (13, 14). In contrast to the situation in *S. sciuri* and *S. vitulinus*, *mecA* was flanked by the regulators *mecR1* and *mecI* in *S. fleurettii*, leading to the hypothesis that the *mec* complex was first assembled in this species (14). Among the other species of the *S. sciuri* group, *Staphylococcus lentus* has been the least studied, and no information at all is available regarding the distribution of SCC*mec* elements in *Staphylococcus stepanovicii*.

A recent study investigating the distribution of *ccr* genes among *S. sciuri* showed that the frequency of *ccr* in methicillin-susceptible *S. sciuri* was much higher (35%) than that described for other coagulase-negative staphylococci (CoNS) (15). Moreover, the most frequent *ccr* allotypes corresponded to homologues of *ccrA3B3*, although homologues of all other *ccr* allotypes were also identified (15). This suggests that *S. sciuri*, besides being the original source of the *mecA* determinant, may also have been the donor of the *ccr* complex for the assembly of SCC*mec*.

Studies on SCC*mec* evolution performed so far suggest an important role of the *S. sciuri* group, but those studies were based only on a limited number of isolates and provided scattered evidence on the evolution, diversification, and assembly of SCC*mec*. In this study, we provide missing links in the evolution of SCC*mec* through the study of a large and diverse collection of isolates belonging to the *S. sciuri* group by the use of whole-genome sequencing.

RESULTS AND DISCUSSION

Studies performed so far suggest an important role of the *S. sciuri* group in SCC*mec* assembly, but when, where, and how the different assembly steps occur is still elusive. In this study, we aim to demonstrate the roles of different species within the *S. sciuri* group in the construction of the cassette. In particular, we were interested in understanding how the *mecA* homologue present in *S. sciuri* evolved to be part of the *mec* complex, how the SCC mobile genetic elements were created, and how the *mec* complex was incorporated into these elements and became mobile. To address these

questions, we sequenced the genome of 106 isolates belonging to *S. sciuri*, *S. vitulinus*, and *S. fleurettii*, all species belonging to the *S. sciuri* group, collected in different time periods, geographic regions, and from both humans and animals, and analyzed the *orfX* region and the region in the vicinity of *mecA* homologues.

To identify proofs of the origin and subsequent evolutionary steps of the different pieces within the SCC*mec* mosaic structure, we looked for the distribution of structural elements of SCC*mec* type III, the most frequent in species of the *S. sciuri* group, using BLAST analysis. Also, we compared the synteny of these structural elements with that of *S. aureus* SCC*mec* III and examined their relatedness using phylogenetic analysis. Finally, to identify blocks of homology between SCC elements in different species, we compared the complete SCC elements using alignment tools and iterative phylogenetic analysis.

Evolution of *mecA* and flanking genes in the native location. The locations of the native *mecA* homologues were identified in all isolates by confirming the presence of genes previously shown to be in the vicinity of *mecA1* in *S. sciuri*, *mecA2* in *S. vitulinus*, and *mecA* in *S. fleurettii* (14). We found that the native location of *mecA* homologues was approximately 200 kb downstream of *orfX* in the three species analyzed. The location of this PBP gene was the closest to *orfX*, compared to the locations of other native PBP genes in *S. sciuri* and other staphylococcal genomes (http://www.ncbi.nlm.nih.gov/) (16–19). This location near *orfX* and *oriC* may have been important for integration of the *mec* complex into the SCC elements by recombination. On the other hand, the integration into a site near *oriC* may have increased the *mecA* gene dosage, providing a potential selective advantage via increased resistance to β-lactams, as previously observed when a strong *mecA1* promoter was formed in *S. sciuri* through the insertion of IS*256* (20).

We also observed that the *mecA* homologues were always flanked by the same genes in the native location, as previously described (14). This included *psm-mec*, a gene implicated in *S. aureus* virulence and located immediately upstream of *mecA* homologues (21), and *ugpQ*, which is located downstream of *mecA* homologues. Interestingly, these genes are exactly the same as those that flank *mecA* in contemporary SCC*mec* types carried by methicillin-resistant *Staphylococcus aureus* (MRSA) (22). However, the level of homology of *psm-mec* and *ugpQ* from MRSA with those from the *S. sciuri* group varied according to the species analyzed. The *psm-mec* and *upgQ* genes most similar to those of MRSA were found in the vicinity of *S. fleurettii* and *S. vitulinus mecA* (98.55% and 99.87 to 100%, respectively), followed by those found in the vicinity of *S. vitulinus mecA2* (97% and 86.56%) and in the vicinity of *S. sciuri mecA1* (91 to 94% and 75 to 85%) (Table 1).

Although most strains contained at least one copy of a *mecA* homologue in the native location, the identities and frequencies of these genes differed in the three species analyzed. *mecA1* was ubiquitous in *S. sciuri* and *mecA* in *S. fleurettii*. Although ubiquity of *mecA1* was previously described in *S. sciuri* (9), the ubiquity of *mecA* in *S. fleurettii* is in contradiction with previous studies, where a much smaller collection of isolates was analyzed (14). In contrast, in *S. vitulinus*, the type of *mecA* homologue found in the native location varied from strain to strain. Half (9/18) of the *S. vitulinus* strains carried *mecA2* (13), but in six *S. vitulinus* strains, we identified a *mecA* homologue with 99% identity to that of *S. aureus mecA*. The remaining three *S. vitulinus* strains had no *mecA* homologue at the native location (Table 1) but instead carried *mecA* near the *orfX* that was 100% identical to *mecA* of MRSA.

In addition to *mecA1*, we found that 16 out of the 76 total *S. sciuri* strains also carried MRSA *mecA* in the *orfX* region. Moreover, the *mecA* homologue most similar to that of MRSA strains was the *mecA* from *S. sciuri* and *S. vitulinus* located at the *orfX* (100% nucleotide identity), followed by *S. fleurettii* and *S. vitulinus mecA* (99% nucleotide identity), *S. vitulinus mecA2* (94%), and *S. sciuri mecA1* (80%), all located at the native location.

TABLE 1 Frequency of genes homologous to those carried by SCC*mec* elements found in *S. sciuri*, *S. vitulinus*, and *S. fleuretti* not containing SCC*mec* at the *orfX*[a]

SCC*mec* element	*S. sciuri*		*S. vitulinus*		*S. fleurettii*	
	Frequency (%)	Homology (%)	Frequency (%)	Homology (%)	Frequency (%)	Homology (%)
J3 region						
ugpQ (J3)	100	75–85	100	87–100	100	99–100
maoC (J3)	63	100	100	100	100	100
pre (J3)	5	64	60	65	17	63
Polypeptide B gene (J3)	2	63	20	99	17	99
mec complex						
IS*431*	47	99	80	99	92	99
IS*1272*	3	63	40	66	25	63
mecA1	100	80				
mecA2			50	90		
mecA			40	99–100	100	97–99
mecR1 and *mecI*			40	99 and 92–99	100	99/92
mecR2	23	78	100	99	100	99
J2 region						
psm-mec (J2)	100	91–94	100	97–99	100	98–99
Gene encoding rhodanese domain-containing protein (J2)	48	77	40	100	100	99
Gene encoding metallo-β-lactamase family protein (J2)	47	78	40	99	100	99
ccr complex						
ccr	38	72–100	13	72–77	8	83
Multiple *ccr* genes	10					
J1 region						
Genes encoding hypothetical proteins (J1)	38	90–100	11	90–100	17	90–100

[a]The nucleotide sequence identities found between these homologues and the ones carried by contemporary SCC*mec* III are also shown.

When *psm-mec*, *upgQ*, and *mecA* homologues from the three species were compared to those of MRSA in a phylogenetic tree (where *S. sciuri* is the most divergent and *S. fleurettii* the most similar to MRSA), the hierarchy observed was comparable to that obtained for other housekeeping genes located far away from the native location and was similar to the phylogenetic hierarchy obtained when housekeeping genes from the different species were used to construct a phylogenetic tree (data not shown). Therefore, the comparison of *mecA* homologues and their neighboring genes to the ones carried by SCC*mec* reflected the phylogeny of the species in an evolutionary perspective (Table 1).

Altogether, these results suggest that the first steps in the evolution of the β-lactam resistance determinant were the evolution of *S. sciuri mecA1* and its neighboring genes into *mecA2* and neighboring gene alleles in *S. vitulinus* and then evolution into *mecA* and neighboring genes in *S. fleurettii*, which are almost identical to those in MRSA. The finding of *S. vitulinus* strains with *mecA* and adjacent genes similar to the ones carried by *S. fleurettii* suggest that after vertical evolution, *mecA* was probably acquired by *S. vitulinus* from *S. fleurettii* by horizontal gene transfer (HGT).

The assembly of the *mec* complex occurs in a stepwise manner along phylogeny. We found that the *mecA* regulators (*mecI* and *mecR1*) in their native locations were always associated with *mecA* in both *S. fleurettii* and *S. vitulinus* but were never found near to *mecA1* and *mecA2*. Whereas the native *mecR1* gene carried by *S. fleurettii* and *S. vitulinus* was 99.6% identical to the *mecR1* in SCC*mec*, the nearby *mecI* gene had a lower identity (92.5%), attributable to a 24-nucleotide difference and alternate stop codon that lengthened the open reading frame (ORF) from 345 to 372 bp. We suggest that the ancestral *mecI* gene was longer and that a deletion of 24 nucleotides occurred during evolution, giving rise to the contemporary *mecI* gene that is found in all SCC*mec* types containing *mec* complex A (5).

We also searched the native location for the presence of the recently described regulatory gene *mecR2*, which encodes an antirepressor of *mecA* and is also part of *mec*

complex A (23). All *S. vitulinus* and *S. fleurettii* strains carried *mecR2*, which is 99% identical to MRSA *mecR2*, near the *mecA* homologue in the native location (*S. vitulinus*, *mecA* and *mecA2*; *S. fleurettii*, *mecA*). In contrast, in *S. sciuri*, we found that only 14 isolates out of 76 (18.42%) carried a *mecR2* homologue in the vicinity of *mecA1* (14 isolates) in the native location. These 14 strains were epidemiologically unrelated, since they were collected from different hosts (including wild animals, production animals, and humans), different geographic origins, and different time periods. The *S. sciuri mecR2* homologue had only 77.56% nucleotide sequence identity with MRSA *mecR2*. This gene may correspond to the ancestral *mecR2* and may be involved in the regulation of *mecA1* expression in *S. sciuri*.

Another element which is part of the *mec* complex and is usually located downstream of *mecA* is the IS*431* element. We observed that this element was absent from the close vicinity of *mecA* homologues in the native locus (data not shown). In contrast, IS*431* was found in the close vicinity of *mecA* when it was located in the *orfX* region within an SCC*mec* element, in *S. sciuri* and *S. vitulinus*. In addition, IS*431* was found with high frequency in all genomes, particularly in *S. fleurettii* genomes (Table 1), but not in the vicinity of *mecA* (data not shown).

These observations suggest that the addition of regulators to a basic *mecA* gene cluster may have occurred in a stepwise manner: *mecR2* was the first gene to be added in *S. sciuri* at the native location near *mecA1*. Although present only in a small fraction of the current *S. sciuri* population, this gene arrangement seems to have been maintained during evolution of the species and to have become ubiquitous in *S. fleurettii* and *S. vitulinus*. Addition of *mecR1* and *mecI* only occurred later, after the evolution of the ancestral *mecA1* into *mecA* was complete. The IS*431* element was probably added only to *mecA* and regulators, after it was mobilized into SCC*mec* or during mobilization, since it was not observed at the native location.

The *ccr* complex is common in *S. sciuri* and is most similar to *ccrA3B3* of SCC*mec* III. We found that the frequency of *ccr* genes was much higher in *S. sciuri* (55%) than in *S. vitulinus* (28%) and *S. fleurettii* (8%) (Table 1). Furthermore, while only *ccrA* and *ccrB* were identified in *S. fleurettii* and *S. vitulinus*, a more diverse pool of *ccr* genes was observed in *S. sciuri*, including *ccrC*, at a 3% frequency, and a new *ccr* type (75% nucleotide identity to *ccrC*), at a 14% frequency, in addition to *ccrA* and *ccrB*. Moreover, *S. sciuri* was the only species in which multiple *ccr* genes were detected in some isolates (10%).

The great majority (70%) of the *ccrA* and *ccrB* allotypes identified in *S. sciuri*, *S. fleurettii*, and *S. vitulinus* were similar to *ccrA3*, *ccrA5*, *ccrB3*, and *ccrB5* (83 to 100% nucleotide identity). These *ccr* allotypes are highly related to *ccrA3B3* carried by SCC*mec* III (*ccrA3*, 85% nucleotide identity with *ccrA5*; *ccrB3*, 83% nucleotide identity to *ccrB5*), as previously observed (15). The types of *ccr* complex were diverse and were represented by different combinations. The most frequent *ccr* complex types observed were *ccrA3B5* (27%), *ccrA5B5* (24%), and *ccrA5B3* (13%). The *ccrA3B5* complex type was observed in *S. sciuri* and *S. vitulinus*, while *ccr* types *ccrA5B5* and *ccrA5B3* were observed exclusively in *S. sciuri*. In addition, *ccrA1B1*, *ccrA1B3*, and *ccrA1B5* were exclusively observed in *S. sciuri* and at low frequency (2% each). The remaining *ccr* types were new, corresponding to combinations of the new *ccrA* and *ccrB* allotypes identified.

In *S. sciuri*, we found the most ancestral alleles of *ccrA* and *ccrB* (see Table S2 in the supplemental material), as well as the combination of *ccrA* and *ccrB* genes that were the most closely related to *ccrA3B3*, which has disseminated into SCC*mec* types carried by MRSA. In addition, the fact that the frequency and diversity of *ccr* genes and complexes are much higher in *S. sciuri* than in *S. vitulinus* and *S. fleurettii* suggests that *S. sciuri* was the most probable donor of the *ccr* complex to form a primordial SCC*mec*.

On the other hand, the high frequency of *ccr* genes, which have a high degree of similarity with the *ccrA3B3* complex in all species of the *S. sciuri* group, supports the hypothesis that SCC elements were transferred within the different species of the *S. sciuri* group and that SCC*mec* III might have been the first cassette to be assembled. Moreover, the existence of *attR* and *attL* sequences within the *orfX* region supports the

statement that *ccr* genes are functional in the *S. sciuri* group of species and are involved in the integration and excision of SCC elements, as has been suggested previously in *S. aureus* (2).

The SCC*mec* J1 region originated in *S. sciuri*, and the J2 and J3 regions originated in *S. fleurettii* and *S. vitulinus*. Besides the two essential elements, the *mec* complex and the *ccr* complex, SCC*mec* also contains three joining regions (J1 to J3), the organization of which within the element may be represented as *orfX*-J3-*mec*-J2-*ccr*-J1-direct repeats (DR).

The J1 region of SCC*mec* III (linking *ccr* and DR) is composed of six different genes encoding hypothetical proteins. We found that at least one, but often two or more, of these genes was present at a higher frequency in *S. sciuri* (38%) than in *S. vitulinus* (11%) or *S. fleurettii* (17%). The similarity of the nucleotide sequences of the genes carried by strains belonging to the *S. sciuri* group and the genes carried by SCC*mec* III was high (90 to 100%). Particularly in *S. sciuri*, two, three, or even six of the genes were found in the same orientation and synteny as in SCC*mec* III of MRSA. Annotation of the vicinity of these genes in all species showed that they were always located in the *orfX* region, and some of them were located inside SCC elements (Table S2). These observations suggest that *S. sciuri* was the most likely donor of the J1 region for the assembly of a primordial SCC*mec*.

The SCC*mec* III J2 genes (linking *mec* and *ccr*) were in general more frequent among *S. fleurettii* and *S. vitulinus* (carrying *mecA*) and less frequent in *S. sciuri*. In particular, genes encoding a rhodanese domain-containing protein and a protein of the metallo-β-lactamase family, located in the distant vicinity of the *mec* complex in SCC*mec*, were found in all three species analyzed. In *S. fleurettii*, they were ubiquitous (100%), and in *S. vitulinus*, they were present only in isolates carrying the *mec* complex (40%) (Table 1). In these species, these genes were located in the vicinity of the *mec* complex, in the native location (*S. fleurettii* and six *S. vitulinus* strains) or in the *orfX* region (three *S. vitulinus* strains). However, in *S. sciuri*, these elements were present either in the vicinity of *mecA1* in the native location or in the vicinity of the *mec* complex near *orfX* (Table 1). When in the native location in the three species, these genes were upstream of *psm-mec*, in the same orientation and synteny as the ones carried by SCC*mec*. In addition, the nucleotide identity of the genes found in *S. sciuri* and the ones carried in SCC*mec* was lower than that of the genes carried by *S. fleurettii* and *S. vitulinus* (Table 1).

Finally, we observed that genes found in J3, namely, genes encoding hypothetical proteins (*pre* and the polypeptide B gene, located upstream of the hypervariable region [HVR]) were more frequent in *S. fleurettii* (*pre*, 17%, and polypeptide B gene, 17%) and *S. vitulinus* (*pre*, 60%, and polypeptide B gene, 20%) but rare in *S. sciuri* (5 to 6% and 2%, respectively) (Table 1). We observed that both genes were always present in the same orientation and synteny as the ones carried by SCC*mec*. However, it was not possible to assess their position relative to the position of *mecA* homologues, since *mecA*, *pre*, and polypeptide B genes were always in different contigs in strains carrying these genes. Similarly, *maoC*, a gene that is part of the hypervariable region located in the J3 region, was located upstream of the *mecA* homologue in the native location in these three species, at different frequencies (*S. fleurettii* and *S. vitulinus*, 100%; *S. sciuri*, 63%).

These results suggest that the SCC*mec* J3 region probably originated in *S. vitulinus* and *S. fleurettii*. However, the genes encoding the rhodanese domain-containing protein and the metallo-β-lactamase family protein of the J2 region, which is located in the vicinity of the *mec* complex, were probably already present in *S. sciuri* and evolved in a manner consistent with the phylogeny and were then mobilized together with the *mec* complex and the more distant J3 regions to form an SCC element. In general, we observed that J regions were much more conserved than the random insertion of genes would dictate, which we believe to result from the following: (i) genetic drift or purifying selection occurring during evolution that could have eliminated all the unnecessary or nonbeneficial genes while keeping those that convey an advantage; (ii) the mobility of SCC elements that might have contributed to the dissemination of the

same genetic content between different strains and species within the same chromosomal location; and (iii) the specificity of recombinase recognition sites, which might have limited the type of elements and genes that can be recombined with SCC.

***S. sciuri* SCC elements carry housekeeping genes and are similar to MRSA SCC*mec*.** SCC structures, bounded by *attR* or *attL*, were observed in all three species, suggesting that the *ccr* genes found were functional. However, the frequency and diversity of such structures were higher in *S. sciuri* than in the other two species. A total of eight different structures were found in the 26 isolates analyzed. In Table S2, there is a detailed description of the variety of SCC non-*mec* elements found in each of the species.

SCC elements carried by *S. sciuri* had in common the presence of genes that confer resistance to metals and, most unexpectedly, genes that are usually associated with housekeeping functions in different staphylococcal species. In *S. sciuri* strains 11/01 and CCUG38359, an SCC non-*mec* element of 54 kb ($SCC_{11/01}$) carrying *ccrA5B5* was identified. This SCC was composed of several elements associated with SCC*mec*, such as a type I restriction-modification system, a cadmium resistance operon, IS*431*, and several genes encoding various hypothetical proteins (Table S2). The nucleotide sequences of these genes were most similar (40 to 100%) to those of genes encoding hypothetical proteins present in SCC*mec* III, SCC*mec* IVa, SCC*mec* IX, and SCC*mec* XI and nontypeable SCC*mec* cassettes carried by *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, and *Staphylococcus pseudointermedius*. Moreover, this element contained ORFs that were similar to genes found in the same region in *ccr*-negative strains. In particular, in the *ccr*-negative strain K22, genes encoding these exact same proteins (100% nucleotide sequence identity) were present 14 kb downstream of *orfX* (Table S3). Furthermore, immediately upstream of the DR, this SCC contained genes encoding proteins that are present elsewhere in the core genome of other staphylococcal species. These genes participate in bacterial central metabolism (encoding glycosyltransferases, oxidases, alcohol dehydrogenases, photolyases, and general stress proteins).

The remaining SCC non-*mec* elements identified in *S. sciuri* were diverse. Strain K116 carried a 36-kb SCC (SCC_{K116}) that contained a *ccr* gene with 75% nucleotide identity with *ccrC*. As with $SCC_{11/01}$, this SCC element carried the *cad* operon and staphylococcal housekeeping genes. Some of these housekeeping genes were similar to those found inside $SCC_{11/01}$ (e.g., genes encoding oxidases, dehydrogenases, and photolyases), but some were different (genes encoding epimerases). Strain SS27 carried a composite SCC element formed by two SCCs: a 45-kb SCC and a 15-kb SCC (both carrying *ccrA1B3* [Table S2]) ($SCC\text{-}CI_{SS27}$). The larger SCC element (SCC_{SS27I}) carried a plasmid with an intact *rep* gene as well as genes conferring resistance to copper, cadmium, and arsenic. The smaller SCC carried a quinone reductase gene as well as the *cad* operon and housekeeping genes, similar to those found in SCC_{K116} (Table S2). These SCC elements also carried genes found inside SCC*mec* cassettes, such as genes encoding hypothetical proteins (61 to 98% nucleotide identity) present in several SCC*mec* cassettes (SCC*mec* II, III, IV, V, IX, X, and XI). In addition, SCC_{SS27I} also carried a rhodanese domain-containing protein and a metallo-β-lactamase family protein typical of the J2 region of SCC*mec* III (Table S2).

In *S. vitulinus* and *S. fleurettii*, the SCC elements found were different from the ones identified in *S. sciuri*. They contained new types of *ccr* and genes encoding mainly hypothetical proteins, some of which had homology with ORFs that are present in SCC*mec* from MRSA. However, in contrast to what was described for *S. sciuri*, housekeeping genes were not found inside *S. vitulinus* or *S. fleurettii* SCC elements. *S. vitulinus* 401946 carried a 20-kb SCC (SCC_{401946}), with a new *ccr* type (*ccr* type 6 [Table S2]). This SCC also carried genes encoding hypothetical proteins identified in SCC*mec* types V and IX, as well as a type I restriction-modification system. In addition, we also found predicted ORFs that had no significant hits in the BLAST server. *S. vitulinus* CH10 carried an 11-kb SCC (SCC_{CH10}), which contained a new *ccr* type (*ccr* type 7 [Table S2]) and genes encoding hypothetical proteins identified in SCC*mec* types IX and XI, as well as predicted ORFs with no significant hits in the BLAST server. *S. fleurettii* 402567 carried

an 18-kb SCC composed of genes encoding hypothetical proteins. Besides the *ccr* genes, which corresponded to new types, the predicted ORFs showed no significant hits in the BLAST server.

Overall, our data showed that SCC structures carried by *S. sciuri* are more similar to the ones seen in contemporary SCC*mec* types, considering both nucleotide sequence identity and synteny. In addition, only *S. sciuri* SCC elements carried housekeeping genes. The frequent inclusion of housekeeping genes inside SCC elements illustrates the level of genomic rearrangement that occurs in the *orfX* region of *S. sciuri*. The benefit to *S. sciuri* of carrying SCC elements made mainly of housekeeping genes is not obvious, but these genes might have originally been a source of genetic diversity and redundancy, which might have helped these primitive species to adapt better and to thrive in their environment. Also, the genes encoding proteins of unknown function contained within these cassettes can convey an unidentified advantage to the strain carrying it, such as access to new environments and hosts or acquisition of new metabolic functions. In this case, the flanking housekeeping genes could be merely coselected and cotransferred and do not necessarily provide a direct benefit to the bacteria. The most probable fate of these housekeeping genes if they were nonbeneficial would be the loss of the genes due to genetic drift or purifying selection. In fact, during evolution, SCC elements like SCC*mec* appear to have specialized and become more efficient, carrying mainly determinants that give rise to a clear advantage to their host, including those associated with antibiotic and metal resistance as well as virulence.

The *orfX* region of *S. sciuri ccr*-negative strains contains genes that are inside SCC non-*mec* and SCC*mec* elements. To further explore the possibility that SCC elements may be assembled from housekeeping genes located near *orfX*, we characterized the *orfX* region of 12 strains not carrying any SCC element or *ccr* complex (seven *S. sciuri*, three *S. fleurettii*, and two *S. vitulinus* strains). We found that this region differed among strains belonging to different species.

Downstream *orfX*, *ccr*-negative *S. sciuri* strains contained mostly genes associated with central metabolism; nevertheless, this region was very diverse with respect to gene content among the strains. This result might indicate that this region of the chromosome frequently undergoes recombination and that genes are being shuffled between strains, due to a high frequency of insertion/excision events. The genes found were not always present, or there were insertions of other genes. Examples of the genes carried by these strains are listed in Table S3 (genes encoding oxidoreductases, serine proteases, alcohol dehydrogenases, epimerases, isomerases, and methyltransferases). A comparison of genes found in this region in *ccr*-negative strains (Table S3) and genes found inside SCC and SCC*mec* carried by *S. sciuri* (Table S2) revealed that some of these genes (with more than 90% nucleotide sequence identity) are found among both structures in the same order and synteny: genes encoding glycosyltransferases, epimerases, dehydrogenases, and oxidases. In addition, in *ccr*-negative strains, genes encoding resistance to cadmium and arsenic were also identified, and these genes were also identified among SCC structures carried by *S. sciuri*.

In some *ccr*-negative *S. sciuri* strains, structural elements typically found inside SCC*mec* could also be identified despite the absence of *ccr* genes in this region of the chromosome. Strains K10, K31, and KLO63 contained genes encoding hypothetical proteins that have been associated with the J1 region of SCC*mec* types IV, V, and IX. In addition, strain K31 carried a truncated DNA restriction-modification system (*hsdR hsdM*) that has been found exclusively in SCC*mec* V.

The fact that housekeeping genes found in *ccr*-negative strains are similar in sequence and synteny to those found in SCC and SCC*mec* elements in *S. sciuri* suggests that a primordial SCC*mec* could have been assembled in this species. Moreover, these observations also suggest that the location of genes near *orfX* was important for their inclusion in an SCC element. Given the fact that the native *mec* location is only 200 kb apart from *orfX*, it is tempting to speculate that a similar type of phenomenon may explain the insertion of the *mec* complex into the SCC element. However, we cannot

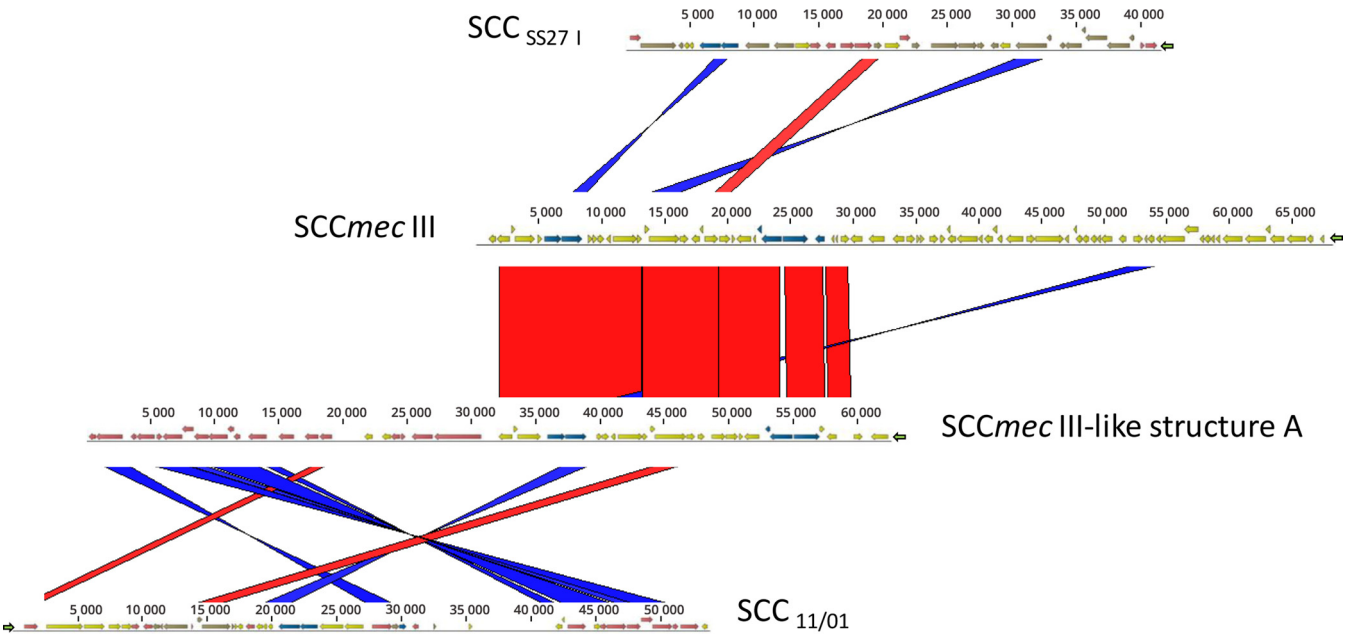

FIG 1 Comparison of regions of homology (highest BLAST score shown in red) between the most frequent SCC*mec* III-like structure A found in *S. sciuri* and *S. vitulinus* with SCC*mec* III carried by MRSA and the SCC non-*mec* element carried by *S. sciuri*, by use of the platform ACT (www.webact.org). A schematic representation of the annotated genes is shown. The core genes are shown in pink, while the genes associated with SCC*mec* are shown in yellow. The *ccrAB* and *mecI*, *mecR1*, and *mecA* genes are highlighted in blue. The *orfX* gene is shown in green. Additional genes which are usually found among staphylococcal mobile genetic elements are depicted in gray (encoding, e.g., heavy-metal resistance-associated genes and plasmid sequences).

disregard the hypothesis that genes found in *ccr*-negative strains near the *orfX* could be remnants of an SCC acquired by *S. sciuri* from another bacterial species. According to current knowledge, the other two bacterial species in which SCC elements could have originated are *Macrococcus caseolyticus*, a species that is phylogenetically related to *Staphylococcus*, and *Enterococcus faecium*; these are the only species, besides staphylococci, in which ancestral forms of the *ccr* genes have been identified (24, 25).

The *orfX* region of *ccr*-negative *S. vitulinus* strains was also diverse but not as much as it was in *S. sciuri* strains. Immediately downstream of *orfX*, three ORFs of unknown function were identified among all *S. vitulinus* strains. In addition, genes encoding proteins that probably participate in central metabolism, such as ATPases, DNA methylases, glycosyltransferases, and acetyltransferases, were also identified. These genes were not identified in all *S. vitulinus* strains. In contrast, the *orfX* region of *S. fleurettii* *ccr*-negative strains was found to be highly conserved, being mainly composed of genes encoding proteins involved in the central metabolism, namely, synthetases and dehydrogenases. In contrast to the results obtained for *S. sciuri*, the SCC non-*mec* elements identified among *S. vitulinus* and *S. fleurettii* strains did not carry any of the genes identified in the *orfX* region of *ccr*-negative strains.

SCC*mec* elements carried by *S. sciuri* are similar but not identical to SCC*mec* III of MRSA. SCC*mec* was identified in *S. sciuri* (16 isolates) and *S. vitulinus* (three isolates) but was absent from all *S. fleurettii* isolates. A total of four different SCC*mec* structures were found. Three of these structures were related to SCC*mec* III (SCC*mec* III-like structures A, B, and C) and one to SCC*mec* type VII, based on the sequence of the genes and their position and structure.

A comparison of the sequence of SCC*mec* III-like elements found in the *S. sciuri* group to the SCC*mec* III of MRSA using WEBACT (www.webact.org) has revealed that the J2 region, the *mec* complex, and the *ccr* complex (approximately 25 kb) were almost exactly the same between the structures in the *S. sciuri* group and SCC*mec* III of MRSA. The structure more closely related to SCC*mec* III and also the most frequent one was SCC*mec* III-like A (Fig. 1). Structure A was found in seven *S. sciuri* isolates (K3, M1640, M1886, JUG1, CH16, CH17, and CH18) and three *S. vitulinus* isolates (H91, CH1, and

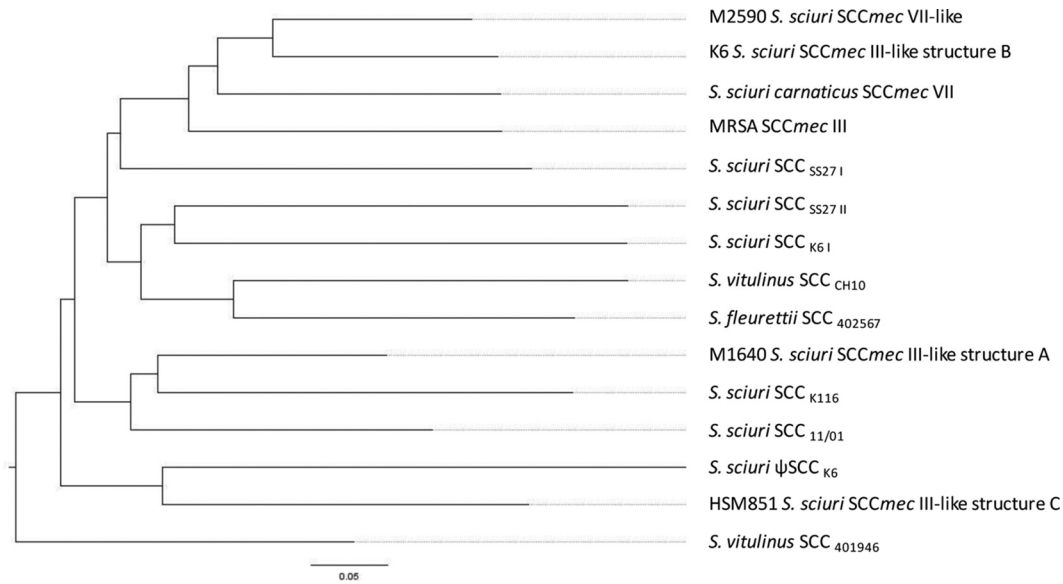

FIG 2 Reconstruction of SCC*mec* and SCC phylogeny with Mauve. *S. sciuri* strains 11/01, HSM851 (partial SCC*mec* cassette), K6, K116, M1640, M2590 (partial SCC*mec* cassette), and SS27, *S. vitulinus* strains CH10 and 401946, and *S. fleurettii* strain 402567 are included.

CH3). This structure (63 kb) was very similar to SCC*mec* III, mainly in the J2 region, containing the same genes encoding hypothetical proteins, the Tn*554* transposase, and the cadmium resistance operon, in addition to the *mec* complex A. The J3 region was very small, encompassing only the HVR region located upstream of the *mec* complex A, which contrasts with the SCC*mec* III J3 region that carries, in addition, plasmid pT181, which contains genes conferring resistance to tetracycline (22). The J1 region was the most divergent between the SCC*mec* III-like A structure and SCC*mec* III. Although some genes encoding hypothetical proteins within this region were common between the two elements, SCC*mec* III-like A contained a more "ancient" *ccr* complex (*ccrA3B5* instead of *ccrA3B3*) (15) and four additional ORFs identified as those encoding a glycosyltransferase, a peptidase, a transcriptional regulator, and a surface protein, which were absent from MRSA SCC*mec* III.

When we compared SCC*mec* III-like A structures with SCC elements identified in our collection, we found several regions of homology between the two. In particular, in the case of genes that were part of the J1 region of the SCC*mec* III-like A element, such as the gene encoding a glycosyltransferase, the same genes were also identified in the J1 region of $SCC_{11/01}$ (Fig. 1). Another example is the *ccrA3B5* that is common to SCC*mec* III-like A and several *S. sciuri* SCC elements. In Fig. 2, there is a tree that resulted from iterative phylogenetic analysis in which the level of relatedness between SCC*mec* and SCC elements can be deduced (Fig. 2) (26). While the SCC*mec* III-like A structure appears to correspond to a primordial form of contemporary SCC*mec* III, the SCC*mec* VII-like and SCC*mec* III-like B structures seem more related to contemporary SCC*mec* types. Interestingly, according to this analysis, SCC*mec* III-like A and C structures, which were present in 63% (12 out of 19) of all strains carrying SCC*mec*, were highly related to the SCC carried by *S. sciuri*.

These results further support the hypothesis that SCC*mec* III-like A structures might have originated in *S. sciuri*, possibly by recombination of the *mec* complex from *S. fleurettii* or *S. vitulinus* with an SCC carried by this species. Moreover, the evolution into contemporary SCC*mec* III probably occurred afterwards and involved mainly a recombination in the J1 region and acquisition of plasmids in the J3 region. Since the *mec* complex that is within *S. aureus* SCC*mec* is usually flanked by insertion sequences (IS), we can speculate that integration of the *mec* complex into an SCC element might have occurred via IS-mediated transposition. However, we did not find in our data any

evidence supporting the possibility that this event occurred in *S. sciuri* species group, since no insertion sequences were found in the vicinity of *mec* homologues or the *mec* complex in the native location in the *S. sciuri*, *S. vitulinus*, and *S. fleurettii* strains analyzed.

SCC*mec* elements are identified in several *S. sciuri* genetic backgrounds. The SNP phylogenetic analysis of the genomes of all isolates (Fig. S1A) showed that *S. sciuri*, *S. vitulinus*, and *S. fleurettii* were separated into three distinct clusters and had an average difference of 150,000 SNPs between their genomes. On the other hand, the SNP analysis of the genomes of isolates belonging to each species independently (Fig. S1B to D) showed that in *S. fleurettii*, the genomes had a range of differences of 3,000 to 9,000 SNPs and that in *S. vitulinus*, the genomes had a range of differences of 4,000 to 7,000 SNPs. In *S. sciuri*, there was a high diversity of genetic backgrounds, which is in accordance with the fact that this species is the most primitive and most widely distributed in nature. Five distinct phylogenetic groups that differed from each other by more than 15,000 SNPs were defined. In this analysis, we included the *S. sciuri* subspecies type strains, K1 (*S. sciuri* subsp. *sciuri*), K3 (*S. sciuri* subsp. *rodentius*), and K11 (*S. sciuri* subsp. *carnaticus*). The genomes of these strains clustered separately, and therefore we suggest that these clusters correspond to the three *S. sciuri* subspecies previously described (27). In Fig. S1B, *S. sciuri* subsp. *sciuri* is depicted as group 2, *S. sciuri* subsp. *rodentius* is depicted as group 4, and *S. sciuri* subsp. *carnaticus* is depicted as group 5. However, we found two additional phylogenetic clusters that probably correspond to two additional *S. sciuri* subspecies (groups 1 and 3).

The *S. sciuri* "new subspecies group 3" was the most represented in our collection, including 30 isolates, followed by *S. sciuri* subsp. *sciuri* ($n = 18$), *S. sciuri* subsp. *rodentius* ($n = 14$), *S. sciuri* subsp. *carnaticus* ($n = 11$), and *S. sciuri* new subspecies group 1 ($n = 3$). The *S. sciuri* new subspecies group 3 isolates were mainly of human origin; *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *carnaticus* isolates were collected mostly from animals (both wild and production animals), and *S. sciuri* subsp. *rodentius* and *S. sciuri* new subspecies group 1 isolates were evenly collected from humans and animals. Overall, groups 1 to 3 were the groups in which diversity in terms of SNP differences was lower (20 to 7,000 SNPs). In groups 4 and 5, the diversity was much higher (100 to 18,000 SNPs).

The frequencies of SCC*mec* and SCC were different among the five groups identified. Specifically, we found that the overall frequency of SCC*mec* and SCC elements was very high in *S. sciuri* new subspecies group 3 and *S. sciuri* subsp. *rodentius* (69 and 71%, respectively) and low in *S. sciuri* subsp. *sciuri* (16.5%). In contrast, *S. sciuri* subsp. *carnaticus* carried exclusively SCC cassettes at a high frequency (45%), and two out of three of the *S. sciuri* new subspecies group 1 isolates carried SCC*mec* elements (67%, 2 out of 3 isolates). Of note, the three subspecies in which SCC*mec* was more frequent (new subspecies groups 1 and 3 and *S. sciuri* subsp. *rodentius*) contained isolates of which most were collected from humans (64 to 77%). Conversely, the phylogenetic groups that comprised isolates with a lower frequency of SCC*mec* (*S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *carnaticus*) were collected mostly from animals (82 to 89%). The difference between these proportions was statistically significant ($P < 0.05$).

The high proportion and distribution of SCC*mec* and SCC elements in diverse *S. sciuri* genetic backgrounds as well as the high proportion of SCC*mec* in the most primitive *S. sciuri* cluster suggest that SCC*mec* was probably assembled in *S. sciuri* a long time ago, possibly before the use of antibiotics in clinical practice. With the wide distribution of *S. sciuri* in nature, it is possible that it has been exposed to β-lactam antibiotics produced by cocolonizing microrganisms in the natural environment, which may have been the driving force for the assembly of SCC*mec*.

In *S. vitulinus*, we did not observe the formation of very defined clusters. However, we found that the genetic backgrounds that carried *mecA2* in the native location were more closely related to each other (average, 4,010 SNPs) than to genetic backgrounds that carried *mecA* in the native locus (average, 6,098 SNPs). We propose that an

ancestral *mecA2* background might have favored the replacement of the *mecA2* locus by *mec* complex A, which evolved divergently, giving rise to two distinct genetic backgrounds. Regarding the events of acquisition of SCC elements, these appear to have occurred several times during the evolution of *S. vitulinus* and were independent of genetic background. Actually, the two SCCs found among *S. vitulinus* were acquired in the genetic background of both strains carrying *mecA2* and those carrying *mecA*. In contrast, SCC*mec* elements were preferentially acquired by strains of the genetic background carrying *mecA2*.

As in the case of *S. fleurettii*, there was a single genetic background that had a higher number of SNP differences than the remaining genetic backgrounds (for CH21, 8,177 SNPs; for the remaining genetic backgrounds, an average of 4,955 SNPs). In addition, this strain was collected in the same country (Switzerland) and during the same time period as the strains of the remaining genetic backgrounds.

Despite a relative high number of isolates included in this study compared to that of previous studies about the *S. sciuri* group, the greatest limitation of this study is that the interpretation of the results is limited to the collection of isolates that we were able to put together.

Conclusion. Our results suggest that SCC*mec* was assembled a long time ago, before the introduction of β-lactam antibiotics into clinical practice. The assembly occurred through several steps involving at least three species of the *S. sciuri* group (Fig. 3).

Our data suggest that the first step in SCC*mec* assembly was the evolution of *mecA1* in its native location together with its flanking genes *psm-mec* and *upgQ*. The second step of evolution was the addition of *mec* regulators. *mecR2* was first acquired by some *S. sciuri* strains and then maintained through phylogeny in *S. vitulinus* and *S. fleurettii*. *mecA* probably first emerged in *S. fleurettii*. The addition of *mecI* and *mecR1* probably occurred in this same species through recombination between *mecA* and adjacent regions with the β-lactamase locus, usually associated with plasmids. The amino acid identities corresponding to genes present in these two loci and the similar orientations and arrangements of the genes suggest an evolutionary link between *mecA* and *blaZ* (28). In addition, it has been demonstrated that the repressor *blaI* can also regulate the expression of *mecA* (29–31). In subsequent steps of evolution, the *mec* complex from *S. fleurettii* may have then been incorporated into the *S. vitulinus* native location by recombination with the *mecA2* locus. We propose that the last donor of *mec* complex A and neighboring regions to an assembled SCC element might have been *S. vitulinus*, since this was the only species in which the *mec* complex and neighboring genes seemed to have been deleted from the native location.

The evolution of SCC elements occurred in parallel with the evolution of the native *mec* locus. The SCC elements most probably originated in *S. sciuri* and were built from housekeeping genes located in the *orfX* region. The integration of the *mec* complex and neighboring genes from *S. vitulinus* into an SCC element probably occurred in *S. sciuri*, since this is the only species in which the same housekeeping genes were found in both SCC and SCC*mec* structures. However, the mechanism that mobilized the *mec* complex from *S. vitulinus* to an SCC in *S. sciuri* is not known.

In *S. sciuri*, an ancestral structure of SCC*mec* III that we refer to as SCC*mec* III-like A was the most frequent SCC*mec* structure found. Our results suggest that (i) SCC*mec* III-like A was the first SCC*mec* cassette and probably emerged in *S. sciuri*, and (ii) SCC*mec* III may have been the first contemporary SCC*mec* type that emerged. In fact, a recent study that focused on the evolution of the pandemic MRSA clone ST239-III, carrying SCC*mec* III, suggests that this clone emerged in the 1960s in Europe (32). Therefore, one can speculate that SCC*mec* III was probably already circulating among the staphylococcal population around that time or even earlier as a means of protection against natural antibiotics produced by microorganisms colonizing the same niche.

Our findings highlight the role of primitive staphylococcal species in the origin of complex pathogenicity islands such as SCC*mec* that, once acquired, can increase in a

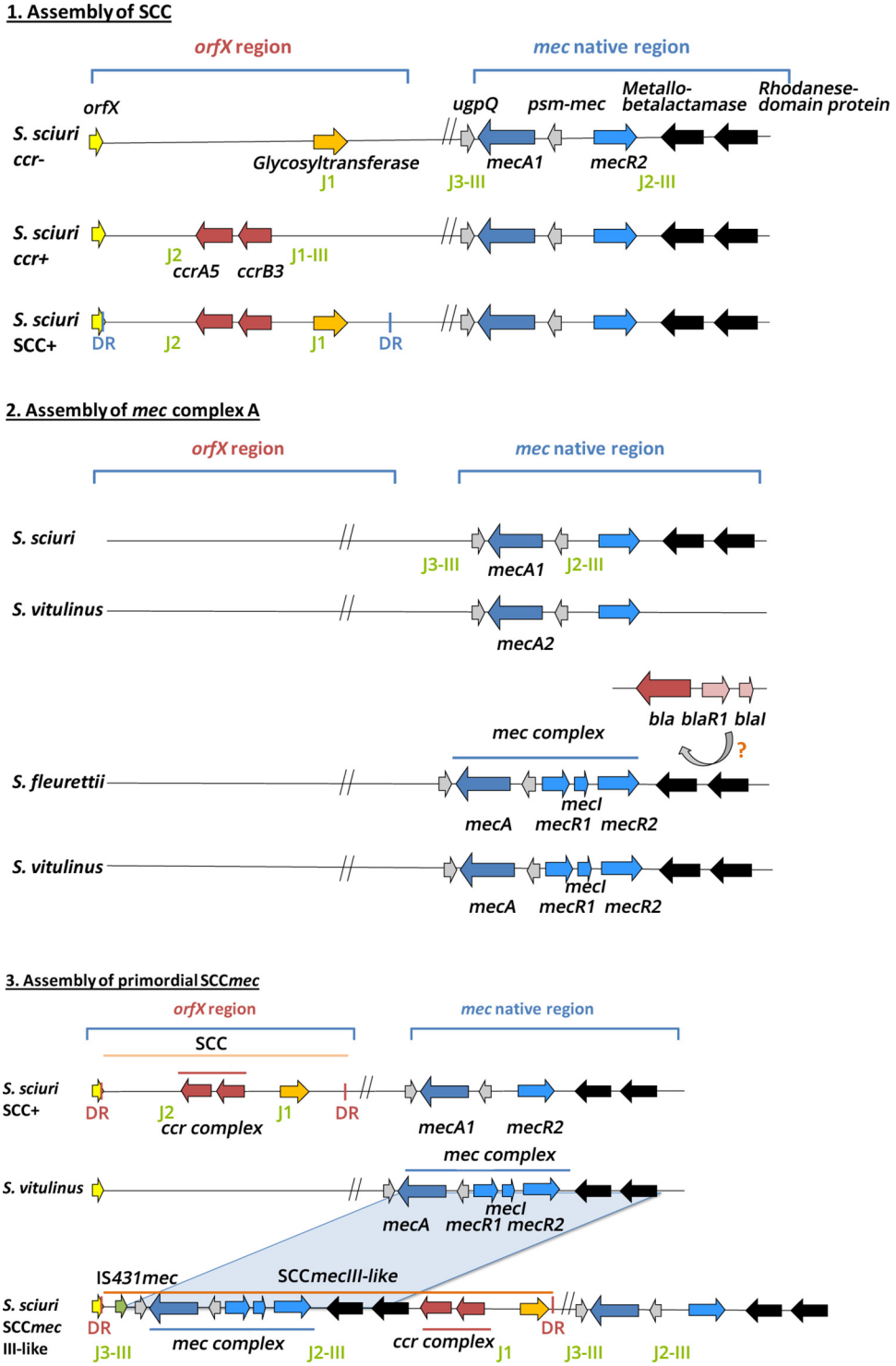

FIG 3 Proposed model for the assembly of the first SCC*mec* structure. The SCC originated in the *S. sciuri* chromosome by the incorporation of housekeeping genes located in *orfX* into SCC elements delimited by *attR* or *attL* and containing the *ccr* complex. On the other hand, *mecA1* and the adjacent regions *psm-mec* and *ugpQ* in the native locus located 200 kb from *orfX* evolved over phylogeny, giving rise to *mecA2* and *mecA* and descendant *psm-mec* and *ugpQ* genes in *S. vitulinus* and *S. fleurettii*, respectively. In *S. fleurettii*, the native locus containing *mecA* might have evolved into a regulatory system like *mec* complex A through recombination with the β-lactamase regulatory locus. This locus would then be incorporated into the *S. vitulinus* chromosome by recombination with the native *mecA2* locus. The *mec* complex A, which would have been donated by *S. vitulinus*, would be integrated in an assembled SCC structure carried by *S. sciuri*, giving rise to a primordial SCC*mec* structure related to SCC*mec* III identified in contemporary MRSA clones.

dramatic way the pathogenic and resistance potential of human colonizing bacteria such as *S. aureus*.

MATERIALS AND METHODS

Bacterial collection. A collection of 106 staphylococcal isolates, comprising 76 *S. sciuri*, 18 *S. vitulinus*, and 12 *S. fleurettii* isolates, was assembled. This is a convenient sample that, according to literature (33–35), represents the species distribution that occurs in nature. In *S. sciuri*, 29 isolates were obtained from humans, and the remaining 47 were recovered from wild and domestic mammals (see Table S1 in the supplemental material). The isolates originated from nine different countries (Czech Republic, Denmark, Portugal, Switzerland, Sweden, the former Yugoslavia, Mozambique, Panama, and the United States) during the period 1972 to 2012. *S. vitulinus* and *S. fleurettii* isolates were collected from horses and bovine mastitis milk samples in Denmark, Switzerland, and the Netherlands in 2004, 2005, and 2010. The *S. sciuri* isolates were identified at the species level by 16S RNA ribotyping and the API-Staph system (bioMérieux, France). *S. fleurettii* and *S. vitulinus* were identified at the species level by sequencing 16S rRNA or *sodA* and MALDI-TOF (matrix-assisted laser desorption ionization–time of flight mass spectrometry) analysis (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) (13, 36, 37). Species identification was confirmed by phylogenetic analysis of the sequence of the *tuf* gene (38) in the sequencing data produced in this study.

DNA preparation and whole-genome sequencing. *S. sciuri* DNA samples were prepared using a phenol-chlorophorm extraction protocol. *S. fleurettii* DNA and *S. vitulinus* DNA were prepared with a DNeasy blood and tissue kit (Qiagen, Limburg, The Netherlands). Sequencing libraries were prepared by sonic fragmentation and adapter ligation and then sequenced on an Illumina HiSeq 2000/2500 platform, producing paired 100-bp reads. The reads were assembled *de novo* using Velvet (39). In addition, *S. fleurettii* strain 402567 was also sequenced using a PacBio RS apparatus and *de novo* assembly was performed using HGAP 3 (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/HGAP-in-SMRT-Analysis).

Assessment of genetic relatedness between isolates. PacBio reads were combined with Illumina reads obtained for *S. fleurettii* isolate 402567 in CLC Genomics Workbench (Qiagen, Limburg, The Netherlands), using the Genome Finishing module. The resulting contigs were ordered using *Staphylococcus xylosus*, which is the species most closely related to *S. fleurettii* that had a closed genome (NCBI accession number CP007208.1; average nucleotide identity with *S. sciuri*, 78%; with *S. vitulinus*, 77.1%; and with *S. fleurettii*, 78.5%). Gaps were closed by mapping Illumina data of the remaining *S. fleurettii* strains to the contigs. The resulting closed genome was annotated with RAST (http://rast.nmpdr.org/). In brief, we found that the *S. fleurettii* 402567 genome was 2.58 Mbp in length, with 31.7% G+C content and 2,498 coding sequences comprising 1,931 putative genes and 567 pseudogenes. The *S. fleurettii* 402567 closed genome was used as a reference to identify single nucleotide polymorphisms (SNPs) in the draft genomes of each subset of strains belonging to a single species, including *S. fleurettii*, *S. vitulinus*, and *S. sciuri*, as well as within the entire collection of samples. Reads were mapped to the reference using Stampy (version 1.0.11), and variants were called using SAMtools (version 0.1.12). Phylogenies were reconstructed using neighbor-joining and drawn in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

BLAST and phylogenetic analysis. The contigs obtained in the Velvet *de novo* assembly were used to assess the presence of several genes of interest, which were detected using BLAST with a 70% nucleotide identity threshold. Sequences of genes found within SCC*mec* III or published gene sequences of *S. sciuri*, *S. fleurettii*, and *S. vitulinus* from NCBI (http://www.ncbi.nlm.nih.gov/) and reference 14 were used as references. The nucleotide sequences of specific genes identified by BLAST analysis were extracted from the sequence of the contigs and then aligned using ClustalW. Phylogenetic trees were constructed using neighbor-joining. In addition, the phylogeny of SCC and SCC*mec* elements was produced by aligning the sequence of these elements with Mauve (26).

Manual annotation. The entire contig containing *orfX* was annotated for a group of arbitrarily chosen strains (17 *S. sciuri*, 4 *S. fleurettii*, and 5 *S. vitulinus* isolates). No specific criteria were used to include the strains in the analysis. *orfX* was identified by BLAST analysis as described above. The remaining ORFs of the contig were identified using GeneMark-hmm (http://exon.gatech.edu/GeneMark/) and annotated with BLAST using the NCBI nonredundant database, with a minimum match threshold of 70% across at least 30% of the gene; *ccr* types were assigned as previously suggested (5).

Comparative genomic analysis. The contigs corresponding to SCC and/or SCC*mec* cassettes containing regions of high identity in nucleotide sequence (>80%) and content were compared using WEBACT (http://www.webact.org/WebACT/home).

Statistical analysis. Statistical significance of differences between proportions was evaluated by the chi-square ($\chi 2$) test using a confidence interval of 95%.

Rationale for the development of the SCC*mec* assembly model. For the construction of the model, we considered that the origin of one gene or block of genes would be the species in which the most ancestral form of the gene was found, the synteny was more conserved, and the frequency and genetic diversity were the highest. On the other hand, the order of occurrence of events was determined by the level of homology with genes present in SCC*mec* III as well as by the presence of intermediate forms of a gene or specific structures in a single phylogenetically defined group, like a species or subspecies, which was also observed in their descendants and not observed in their ancestors. Also, when a similar structure was found in different phylogenetically defined groups, this would suggest the occurrence of horizontal gene transfer (HGT) between them.

Accession number(s). The raw reads of the 106 isolates analyzed in this study and the closed genome of *S. fleurettii* were deposited in the ENA database under accession number PRJEB18761.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02302-16.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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REFERENCES

1. Hartman BJ, Tomasz A. 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus. J Bacteriol 158:513–516.
2. Katayama Y, Ito T, Hiramatsu K. 2000. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother 44:1549–1555. https://doi.org/10.1128/AAC.44.6.1549-1555.2000.
3. Chambers HF, Deleo FR. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol 7:629–641. https://doi.org/10.1038/nrmicro2200.
4. CDC. 2013. Antibiotic resistance threats in the United States, 2013. U.S. Centers for Disease Control and Prevention, Atlanta, GA. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf.
5. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). 2009. Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother 53:4961–4967. https://doi.org/10.1128/AAC.00579-09.
6. Boundy S, Safo MK, Wang L, Musayev FN, O'Farrell HC, Rife JP, Archer GL. 2013. Characterization of the Staphylococcus aureus rRNA methyltransferase encoded by orfX, the gene containing the staphylococcal chromosome cassette mec (SCCmec) insertion site. J Biol Chem 288:132–140. https://doi.org/10.1074/jbc.M112.385138.
7. Ito T, Katayama Y, Hiramatsu K. 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob Agents Chemother 43:1449–1458.
8. Misiura A, Pigli YZ, Boyle-Vavra S, Daum RS, Boocock MR, Rice PA. 2013. Roles of two large serine recombinases in mobilizing the methicillin-resistance cassette SCC*mec*. Mol Microbiol 88:1218–1229. https://doi.org/10.1111/mmi.12253.
9. Couto I, de Lencastre H, Severina E, Kloos W, Webster JA, Hubner RJ, Sanches IS, Tomasz A. 1996. Ubiquitous presence of a mecA homologue in natural isolates of Staphylococcus sciuri. Microb Drug Resist 2:377–391. https://doi.org/10.1089/mdr.1996.2.377.
10. Wu S, Piscitelli C, de Lencastre H, Tomasz A. 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of mecA from a methicillin susceptible strain of Staphylococcus sciuri. Microb Drug Resist 2:435–441. https://doi.org/10.1089/mdr.1996.2.435.
11. Wu SW, de Lencastre H, Tomasz A. 2001. Recruitment of the mecA gene homologue of Staphylococcus sciuri into a resistance determinant and expression of the resistant phenotype in Staphylococcus aureus. J Bacteriol 183:2417–2424. https://doi.org/10.1128/JB.183.8.2417-2424.2001.
12. Antignac A, Tomasz A. 2009. Reconstruction of the phenotypes of methicillin-resistant Staphylococcus aureus by replacement of the staphylococcal cassette chromosome mec with a plasmid-borne copy of Staphylococcus sciuri pbpD gene. Antimicrob Agents Chemother 53:435–441. https://doi.org/10.1128/AAC.01099-08.
13. Schnellmann C, Gerber V, Rossano A, Jaquier V, Panchaud Y, Doherr MG, Thomann A, Straub R, Perreten V. 2006. Presence of new mecA and mph(C) variants conferring antibiotic resistance in Staphylococcus spp. isolated from the skin of horses before and after clinic admission. J Clin Microbiol 44:4444–4454. https://doi.org/10.1128/JCM.00868-06.
14. Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K. 2010. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother 54:4352–4359. https://doi.org/10.1128/AAC.00356-10.
15. Rolo J, de Lencastre H, Miragaia M. 2014. High frequency and diversity of cassette chromosome recombinases (ccr) in methicillin-susceptible Staphylococcus sciuri. J Antimicrob Chemother 69:1461–1469. https://doi.org/10.1093/jac/dku028.
16. Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG,

Spratt BG, Parkhill J. 2004. Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 101:9786–9791. https://doi.org/10.1073/pnas.0402521101.
17. Cavanagh JP, Hjerde E, Holden MT, Kahlke T, Klingenberg C, Flægstad T, Parkhill J, Bentley SD, Sollid JU. 2014. Whole-genome sequencing reveals clonal expansion of multiresistant Staphylococcus haemolyticus in European hospitals. J Antimicrob Chemother 69:2920–2927. https://doi.org/10.1093/jac/dku271.
18. Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, Chantratita N, Gardete S, Tavares A, Day N, Lindsay JA, Edgeworth JD, de Lencastre H, Parkhill J, Peacock SJ, Bentley SD. 2010. Evolution of MRSA during hospital transmission and intercontinental spread. Science 327:469–474. https://doi.org/10.1126/science.1182395.
19. Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Weidman J, Tran K, Kang K, Hance IR, Nelson KE, Fraser CM. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. J Bacteriol 187:2426–2438. https://doi.org/10.1128/JB.187.7.2426-2438.2005.
20. Couto I, Wu SW, Tomasz A, de Lencastre H. 2003. Development of methicillin resistance in clinical isolates of Staphylococcus sciuri by transcriptional activation of the mecA homologue native to the species. J Bacteriol 185:645–653. https://doi.org/10.1128/JB.185.2.645-653.2003.
21. Queck SY, Khan BA, Wang R, Bach TH, Kretschmer D, Chen L, Kreiswirth BN, Peschel A, Deleo FR, Otto M. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog 5:e1000533. https://doi.org/10.1371/journal.ppat.1000533.
22. Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K. 2001. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 45:1323–1336. https://doi.org/10.1128/AAC.45.5.1323-1336.2001.
23. Arêde P, Milheiriço C, de Lencastre H, Oliveira DC. 2012. The anti-repressor MecR2 promotes the proteolysis of the mecA repressor and enables optimal expression of β-lactam resistance in MRSA. PLoS Pathog 8:e1002816. https://doi.org/10.1371/journal.ppat.1002816.
24. Tsubakishita S, Kuwahara-Arai K, Baba T, Hiramatsu K. 2010. Staphylococcal cassette chromosome mec-like element in Macrococcus caseolyticus. Antimicrob Agents Chemother 54:1469–1475. https://doi.org/10.1128/AAC.00575-09.
25. Bjørkeng EK, Tessema GT, Lundblad EW, Butaye P, Willems R, Sollid JE, Sundsfjord A, Hegstad K. 2010. ccrABEnt serine recombinase genes are widely distributed in the Enterococcus faecium and Enterococcus casseliflavus species groups and are expressed in E. faecium. Microbiology 156:3624–3634. https://doi.org/10.1099/mic.0.041491-0.
26. Darling AC, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14:1394–1403. https://doi.org/10.1101/gr.2289704.
27. Kloos WE, Ballard DN, Webster JA, Hubner RJ, Tomasz A, Couto I, Sloan GL, Dehart HP, Fiedler F, Schubert K, de Lencastre H, Sanches IS, Heath HE, Leblanc PA, Ljungh A. 1997. Ribotype delineation and description of Staphylococcus sciuri subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. Int J Syst Bacteriol 47:313–323. https://doi.org/10.1099/00207713-47-2-313.
28. Song MD, Wachi M, Doi M, Ishino F, Matsuhashi M. 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant Staphylococcus aureus by gene fusion. FEBS Lett 221:167–171. https://doi.org/10.1016/0014-5793(87)80373-3.
29. Sabat AJ, Pournaras S, Akkerboom V, Tsakris A, Grundmann H, Friedrich AW. 2015. Whole-genome analysis of an oxacillin-susceptible CC80 mecA-positive Staphylococcus aureus clinical isolate: insights into the mechanisms of cryptic methicillin resistance. J Antimicrob Chemother https://doi.org/10.1093/jac/dkv210.
30. Cohen S, Gibson CJ, Sweeney HM. 1972. Phenotypic suppression of methicillin resistance in Staphylococcus aureus by mutant noninducible penicillinase plasmids. J Bacteriol 112:682–689.
31. Hackbarth CJ, Chambers HF. 1993. blaI and blaR1 regulate beta-lactamase and PBP 2a production in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 37:1144–1149. https://doi.org/10.1128/AAC.37.5.1144.
32. Castillo-Ramírez S, Corander J, Marttinen P, Aldeljawi M, Hanage WP, Westh H, Boye K, Gulay Z, Bentley SD, Parkhill J, Holden MT, Feil EJ. 2012. Phylogeographic variation in recombination rates within a global clone of methicillin-resistant Staphylococcus aureus. Genome Biol 13:R126. https://doi.org/10.1186/gb-2012-13-12-r126.
33. Nemeghaire S, Argudín MA, Feßler AT, Hauschild T, Schwarz S, Butaye P. 2014. The ecological importance of the *Staphylococcus sciuri* species group as a reservoir for resistance and virulence genes. Vet Microbiol 171:342–356. https://doi.org/10.1016/j.vetmic.2014.02.005.
34. Nagase N, Sasaki A, Yamashita K, Shimizu A, Wakita Y, Kitai S, Kawano J. 2002. Isolation and species distribution of staphylococci from animal and human skin. J Vet Med Sci 64:245–350.
35. Stepanovic S, Dakic I, Morrison D, Hauschild T, Jezek P, Petrás P, Martel A, Vukovic D, Shittu A, Devriese LA. 2005. Identification and characterization of clinical isolates of members of the *Staphylococcus sciuri* group. J Clin Microbiol 43:956–958. https://doi.org/10.1128/JCM.43.2.956-958.2005.
36. Poyart C, Quesne G, Boumaila C, Trieu-Cuot P. 2001. Rapid and accurate species-level identification of coagulase-negative staphylococci by using the sodA gene as a target. J Clin Microbiol 39:4296–4301. https://doi.org/10.1128/JCM.39.12.4296-4301.2001.
37. Frey Y, Rodriguez JP, Thomann A, Schwendener S, Perreten V. 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. J Dairy Sci 96:2247–2257. https://doi.org/10.3168/jds.2012-6091.
38. Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M, Bergeron MG. 2001. Development of a PCR assay for identification of staphylococci at genus and species levels. J Clin Microbiol 39:2541–2547. https://doi.org/10.1128/JCM.39.7.2541-2547.2001.
39. Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18:821–829. https://doi.org/10.1101/gr.074492.107.